Clean copy of changes to specification – 09/853,753

Page 4, line 27 to 30, change to read:

In another aspect, this invention comprises a substantially pure mammalian GPI-anchored small leucine-rich proteoglycan, represented by the sequence of amino acids set forth in Figure 7 (SEQ ID NO:2); SEQ ID NO:2 having at least one conservative amino acid substitution; or an amino acid sequence which is at least 50% homologous to SEQ ID NO:2.

Page 5, lines 29 and 30 and page 6, lines 1 and 2, change to read:

Figure 1: Phenotype of complete X-linked CSNB. Figure 1A: The electroretinogram of a patient compared with an age-matched normal. Figure 1B: Rod and L/M cone sensitivities measured on a 12 degree grid across the visual field of a patient. Figure 1C: Spectral sensitivity measurements, dark-adapted, in a representative normal subject (open squares) and the patient (filled triangles).

Page 6, lines 11 to 22, change to read:

Figure 3A: Physical map of the CSNB1 minimal region indicating the location of overlapping BACs and PACs (short lines) and the chromosomal position of several genes in this region, including *NYX*. The lower horizontal line demonstrates the genomic organization of *NYX*, showing that it is comprised of three exons, with a translation start site in the second exon, a stop codon in the third exon and a polyadenylation sequence in the 3' untranslated region. Figure 3B: The amino acid sequence of nyctalopin shows homology with members of the SLRP family of proteins. The protein has 11 leucine-rich repeat motifs with a 24 amino acid consensus for small leucine-rich proteoglycans with cysteine clusters flanking the repeat core of the protein. The conserved amino acids are shown in bold. Figure 3C: Dendogram showing the predicted relationship among members of SLRP. Chondroadherin (CHAD) and nyctalopin appear to represent a fourth class (IV) within SLRP. Figure 3D: Homology comparison of NYX and CHAD using LALIGN™ found these genes to be 30.8% identical and 57.5% similar.

Page 6, lines 24 to 29 and page 7 lines 1 and 2, change to read:

A7

Figure 4: cDNA expression profile of *NYX* in various human tissues. Figure 4A: Upper panel shows a 755 bp fragment of the *NYX* mRNA in retina and kidney tissue samples. Lower panel shows the 281 bp fragment of EST JRL4A1 which serves as a positive control. Figure 4B: Using a NYX-antisense digoxigenin-labelled riboprobe (NYX-AS) for i*n situ* hybridization in human retinal sections showing the expression of *NYX* in the inner segment (IS) of photoreceptors, in the outer- and inner-nuclear layers (ONL, INL), and in the ganglion cell layer (GCL). Figure 4C: No significant staining was observed using a NYX-sense probe. Figure 4D: Rhodopsin – antisense probe (RHO-AS) labelled rod photoreceptors in the IS and ONL.

Page 7, lines 4 to 18, change to read:

A8

Figure 5A: Identification of a putative nonsense mutation in families 610 and 620 in exon 3 (at nucleotide 1049) caused by a G to A transition, which changes a Trp to a stop codon. Segregation analysis of this mutation was performed by restriction endonuclease digestion. Affected individuals show the loss of a *Fok*I restriction site, female carriers have fragments indicating both the presence and absence of this restriction site, and unaffected males show only the *Fok*I site. Figure 5B: Identification of a 24-bp deletion observed in seven (six shown) different families. This mutation results in the loss of eight amino acids beginning at codon 29. The segregation of the 24-bp deletion was performed by PCR amplification of genomic DNA and the products were subjected to agarose gel electrophoresis. The presence of the smaller PCR fragment representing the deletion was observed in affected males and carrier females. Figure 5C: Identification of an insertion mutation of 21 nt between nucleotides 444 and 445 in patient 650-1, which results in the addition of seven amino acids to the protein. Figure 5D: Identification of a missense mutation in patient P520-IV-27 caused by a T to A transversion at nucleotide 638, which changes a Leu codon to a Gln codon.

Page 7, lines 25 and 26, change to read:

A9

Figure 7: Nucleotide sequence of human *NYX* (SEQ ID NO:1) with the amino acid sequence of nyctalopin in single letter code underneath (SEQ ID NO:2).

Page 8, lines 13 to 21, change to read:

A10

The term "carrier" refers to a female who is heterozygous for a single recessive gene and does not have the phenotype associated with complete X-linked CSNB. The terms "isolated" or "substantially pure" nucleic acid or polynucleotide (e.g., an RNA, DNA or a mixed polymer) is one that is substantially separated from other cellular components that naturally accompany a native human sequence or protein, e.g., ribosomes, polymerases, many other human genome sequences and proteins. The term embraces a nucleic acid sequence that has been removed from its naturally occurring environment, and includes recombinant or cloned DNA isolates and chemically synthesized analogs or analogs that are biologically synthesized by heterologous systems.

Page 15, lines 6 to 16, change to read:

A11

To position genetic markers accurately across the CSNB1 minimal region and identify candidate genes for the CSNB1 locus, a robust physical map of the CSNB1 minimal region in Xp11.4 may be developed. A subset of BAC and PAC clones from the minimal tiling path of the estimated 1.2 Mb CSNB1 minimal region is sequenced to between 1-2.7-fold redundancy. A sub-library is constructed [20] for each of the BAC clones and random clones from each sub-library are sequenced with the aid of ABI 373 or ABI 377 sequencing machines and fluorescently labeled primers (ABI, Amersham). DNAStar™ software is used for gel trace analysis and contig assembly as well as DNA and protein alignments. DNA and protein sequences are then examined against available public databases using the various Blast™ programs available through the network server at the National Center for Biotechnology Information. A novel open reading frame is present in this region, which the Applicant has designated to be the *NYX* gene.

Page 15 lines 28 to 30 and page 16 lines 1 to 8, change to read:

A17

The extended *NYX* cDNA sequence is established by sequencing of PCR and RACE products using first-strand cDNA from total human retinal RNA as the template for PCR. Touchdown PCR using the Failsafe™ PCR Premix Selection Kit is carried out according to the manufacturer's protocol (Epicentre Technologies). RACE is carried out using Human Retina cDNA Marathon-Ready™ cDNA (Clontech). A secondary amplification using a nested *NYX* primer is performed. RACE and PCR products are gel purified using the Concert™ Gel Extraction kit (Life Technologies) and sequenced using the ThermoSequenase™ P-radiolabelled terminator cycle sequencing kit (Amersham Life Science). To establish the genomic organization of the *NYX* gene, the full-length cDNA sequence of *NYX* is compared to the genomic sequence derived from our analysis of BAC clone 378P5 and that produced by the Sanger Centre for BAC clone 169I5.

Page 16, line 20 to 30 and page 17 lines 1 to 15, change to read:

NYX encodes a 481 amino acid protein, herein called nyctalopin, which has sequence similarity with members of the superfamily of proteins containing tandem arrays of the leucine-rich repeat (LRR) motif [10,13]. Such proteins are known to function in protein-protein interactions, especially in matrix assembly, and therefore nyctalopin may possibly be mediating specific neural connections between cells in the retina. Moreover, the presence of the 24 amino acid consensus: x-x-I/V/L-x-x-x-x-F/P/L-x-x-L/P-x-x-L-x-x-L/I-x-L-x-x-N-x-I/L (where I,V,L,F,P and N are single letter amino acid codes and "x" represents any amino acid) in the core protein with cysteine clusters flanking the LRR domain (see Figure 3B), qualifies nyctalopin as a new member of the subfamily of small leucine-rich proteoglycans (SLRPs) [10]. From a homology comparison of nyctalopin with other SLRP proteins, it is evident that nyctalopin is a unique member of this subfamily and the LRR superfamily in general. Nyctalopin has five putative consensus sequences (N-X-(S/T)) necessary for substitution by N-linked oligosaccharides or keratan sulfate [14], three of these sequences lie within the LRR region. The

NH_2-terminal end of nyctalopin is predicted [15] to contain a membrane signal peptide with a putative cleavage site between amino acid 23 and 24, AWA-VG (Figure 3). In addition, the carboxyl-terminal region of nyctalopin contains a GPI-anchor signal sequence, including the requisite GPI N-terminal signal sequence (amino acids 339 to 379), the C-terminal hydrophobic region (last 22 amino acids) and a potential cleavage site at amino acids 445-447 [16] (Fig. 3B). The identification of these sites was accomplished at the website www.expasy.ch/tools, and is well known to those skilled in the art. Thus, *NYX* codes for a GPI-anchored proteoglycan with a putative membrane signal peptide. Without being limited to a theory, these results suggest that the clinical features of complete X-linked CSNB can be explained by the presence of a mutant nyctalopin (or entire absence of nyctalopin) causing the disruption of selected connections or interactions between retinal neurons, including those of the retinal ON-bipolar pathway, possibly during early stages of embryonic development.

Page 18, lines 17 to 22, change to read:

Expression of foreign sequences in bacteria such as *E. coli* requires the insertion of the sequence into a vector, usually a plasmid, which contains several elements such as sequences encoding a selectable marker genes, a controllable transcription promoter, translation control sequences and a polylinker. A relatively simple vector that can be used in *E. coli* is pBluescript™ which utilizes the lac promoter and a neighbouring lacZ gene whose function is disrupted when the foreign gene sequence is inserted into the vector.

Page 22, lines 1, change to read:

(o) allele-specific oligonucleotide (ASO) analysis.

Page 22 lines 28 to 30 and page 23 lines 1 to 10, change to read:

Fourteen different mutations have been identified in *NYX*, none of which are observed in chromosomes from normal individuals. In nyctalopin, there are 11 leucine-rich repeats, which

are all highly conserved with respect to the consensus sequence in SLRPs, and these are flanked by cysteine clusters (see Figure 3B) [10]. The deletion of a portion of the cysteine cluster in the amino-terminal portion of nyctalopin appears to be responsible for complete X-linked CSNB in six families, which highlights the importance of this conserved region. The mutation that causes a stop codon on the carboxyl-terminal side of the leucine-rich repeats and another cysteine cluster, likely affects the ability of the protein to anchor in the membrane, as the protein portion on the carboxyl-terminal side of this mutation is presumed to be important for GPI anchoring nyctalopin in the cellular membrane. Mutations that replace a consensus amino acid with another amino acid are presumed to disrupt an essential amino acid function. Mutations that result in the insertion (or deletion) of amino acids in the protein are presumed to alter the folding of the protein

Page 23, lines 20 to 28, change to read:

Full length cDNA clones are constructed by a variety of methods known to those skilled in the art. Such methods include screening a cDNA library with a labeled DNA probe of the gene of interest, identifying overlapping cDNA clones and ligating them together into one clone that contains the entire coding region. One can also obtain a full length cDNA clone in one step from a library, obviating the need to perform intermediary ligation steps. If the 5'or 3' end only of the clone is missing, methods such as RACE (rapid amplification of cDNA ends) are used to complete the sequence, or if the full length sequence is known, PCR amplification and ligation of the fragments onto the ends of the cDNA clone may be used. The cDNA product is cloned into the vector of choice, such as a pUC™ vector or pBluescript™.

Page 25, lines 20 to 29, change to read:

Twenty-four families with complete X-linked CSNB were included in this study. The diagnosis of complete X-linked CSNB in these families involved electrophysiological and psychophysical testing [18], which established the reduction or alteration of the rod pathway-

and cone pathway-mediated function in the retina of these patients. The results of this type of testing on normal and affected subjects is shown in Figure 1. Figure 1A shows that the virtual absence of a rod response, the relative preservation of the scotopic white-flash a-wave with a severely subnormal b-wave, and the loss of the first two major photopic oscillatory potentials for thephotopic single flash. Figure 1B shows the loss of rod sensitivity across the retina, and scattered loss of cone sensitivity. Figure 1C the spectral sensitivity measurements, dark adapted, in a normal subject and a patient.

Page 26, lines 16 to 21, change to read:

Using these markers and other known markers, an analysis of selected recombinant X chromosomes in the set of families with complete X-linked CSNB enable us to moved the distal boundary of the CSNB1 minimal region from DXS556 to the interval between 200L4CA1 and DXS8012 (Fig. 2 and Fig. 3A). From further analysis, the proximal crossover previously observed in patient V:1 in family P23 [19] was limited to the interval between DXS1207 and DXS228 (Fig. 2 and Fig. 3A).

Page 26, lines 25 to 30 and page 27 lines 1 to 8, change to read:

To position genetic markers accurately across the CSNB1 minimal region and identify candidate genes for the CSNB1 locus, a robust physical map of the CSNB1 minimal region in Xp11.4 was developed. A subset of BAC and PAC clones from the minimal tiling path of the estimated 1.2 Mb CSNB1 minimal region is shown in Figure 3. BAC clones 378P5, 36P21, 160H17, and 317C4, which had not been sequenced at the Sanger Center, were sequenced to between 1-2.7-fold redundancy to identify additional candidate genes. A sub-library was constructed [20] for each of the BAC clones. DNA from each BAC was isolated, randomly sheared by nebulization, and fractionated by agarose gel electrophoresis. Fragments (2-4 kb) were collected, blunt-ended, and cloned into M13mp19 using standard techniques. Random clones from each sub-library were sequenced with the aid of ABI 373 or ABI 377 sequencing machines and fluorescently labeled primers (ABI, Amersham). DNAStar™ software was used for gel trace analysis and contig assembly as well as DNA and protein alignments. DNA and

protein sequences were examined against available public databases using the various Blast™ programs available through the network server at the National Center for Biotechnology Information.

Page 27, lines 10 to 23, change to read:

The extended *NYX* cDNA sequence was established by sequencing of PCR and RACE products. First-strand cDNA from total human retinal RNA was used as the template for PCR. Touchdown PCR using the Failsafe™ PCR Premix Selection Kit was carried out according to the manufacturer's protocol (Epicentre Technologies). RACE was carried out using Human Retina Marathon-Ready™ cDNA (Clontech). As the GC content of exon 3 of *NYX* averages 72%, Advantage-GC 2™ Polymerase (Clontech) was used, and touchdown PCR for 5' RACE (94°C for 30s, 72°C for 4 min. for 5 cycles; 94°C for 30s, 70°C for 4 min. for 5 cycles; 94°C for 30s, 68°C for 4 min. for a further 25 cycles). A secondary amplification using a nested *NYX* specific primer was then performed using the same conditions as above. RACE and PCR products were gel purified using the Concert™ Gel Extraction kit (Life Technologies) and sequenced using the ThermoSequenase™ ^{33}P-radiolabelled terminator cycle sequencing kit (Amersham Life Science). To establish the genomic organization of the *NYX* gene, the full-length cDNA sequence of *NYX* was compared to the genomic sequence derived from our analysis of BAC clone 378P5 and that produced by the Sanger Centre for BAC clone 169I5.

Page 27, lines 25 to page 28, lines 1 and 2, change to read:

BAC clone 378P5 (Fig. 3A) yielded a sequence that had partial complete homology with a 526-bp expressed sequence tag Q14392 (Accession No. AI861796). Sequence of the BAC clone 378P5 in the region of homology to ESTQ14392 overlaps with the partial DNA sequence from BAC clone 169I5(Fig. 3(a)). GenScan™ and GeneFinder™ analysis of a 20 kb portion of the genomic sequence from clone 169I5 (Sanger Centre, www.sanger.ac.uk) that encompasses EST Q14392 predicted a novel open reading frame which we have designated *NYX* and is shown in Figure 3A.

Page 28, lines 22 to 30 and page 29, lines 1 to 6, change to read:

The RNA *in situ* hybridization method was performed as described [21-23]. Sections were hybridized with 668-bp antisense probe (from nt1557-2224 of the *NYX* cDNA), at a concentration of 400 ng/ml. The hybridization was done in 50% formamide, 5 x sodium chloride-sodium citrate-phosphate (SSCP) and 40 mg/ml salmon sperm DNA, for 18 hr at 65°C under Parafilm™ (American National Can, Chicago, IL). Two post-hybridization washes were washes were performed with 2 x SSCP for a total of 30 min at 68°C; followed by two washes in 0.1 x SSCP for a total of 1 hr at 68°C. Incubation with Fab fragments from an anti-digoxigenin antibody (1:5000) from sheep, conjugated with alkaline phosphatase (Boehringer Mannheim) for 2 hr at 22°C, followed by washes with SSCP, was used for the detection of the digoxigenin-labeled riboprobes. Precipitation of the reaction products of nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Life Technologies) continued for 3–5 days with a daily change of substrate solution. Slides were mounted with Glycergel™ (Dako, Mississauga, Ontario, Canada). Images were obtained digitally on a Carl Zeiss Axioskop 2™ microscope with a cooled CCD camera (Diagnostic Instruments, Inc.). The images were converted to a grey scale, and the brightness and contrast were adjusted in Adobe Photoshop™.

Page 30, lines 6 to 12, change to read:

Six other families were found to have an in-phase 24-nt deletion that results in the loss of eight amino acids - RACPAACA (see Figure 5B). Six of these amino acids form part of a conserved cysteine-cluster on the amino-terminal side of the leucine-rich repeats, as shown in Figure 3B. Haplotype analysis of X chromosomes with this deletion mutation from each of the six families revealed nearly identical haplotypes, suggesting that these families share a common founder mutation. In three families, insertion mutations representing duplications of adjacent protein sequence add either six or three amino acids (Figure 3B).

Page 30, lines 14 to 18, change to read:

Single-nucleotide changes in seven other families with complete X-linked CSNB are predicted to cause missense mutations. Four of these mutations change a conserved leucine to a glutamine or proline; one changes a conserved Asparagine to a Lysine and another changes a conserved Phenylalanine to a Serine (Figure 6). Figure 5D demonstrates one of these particular missense mutations, a T to an A which results in a Leucine to Glutamine change.

Page 31, lines 7 to 16, change to read:

Segregation analysis can also be performed, as shown in Figure 5B. Primers were designed as described above to amplify over the region where the 8-amino acid deletion occurs. The primers used were:

Forward: GATTTTTCCTGGGGTGACCT (SEQ ID NO:13)

Reverse: GTCCAGGTCGATGGAGACC (SEQ ID NO:14)

PCR was used to amplify the region of interest from genomic DNA of affected, non-affected and carrier individuals. The PCR products were electrophoresed through agarose gels. In Figure 5C the gene carrying the deletion was identified in affected and carrier individuals by a 238 bp PCR fragment. The PCR fragment from normal, non-deleted chromosomes was 262 bp.